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CODEN: 69CAAD

Thanks

Journal of Cell Science 112, 435-445 (1999)
Printed in Great Britain The Company of Biologists Limited 1999
JCS4639

Induction of vascular smooth muscle cell tenascin-C gene expression by denatured type I collagen is dependent upon a β3 integrin-mediated mitogen-activated protein kinase pathway and a 122-base pair promoter element

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Accepted 7 December 1998; published on WWW 25 January 1999

SUMMARY

Tenascin-C is an extracellular matrix glycoprotein, the expression of which is upregulated in remodeling arteries. In previous studies we showed that the presence of tenascin-C alters vascular smooth muscle cell shape and amplifies their proliferative response by promoting growth factor receptor clustering and phosphorylation. Moreover, we demonstrated that denatured type I collagen induces smooth muscle cell tenascin-C protein production via β3 integrins. In the present study, we examine the pathway by which β3 integrins stimulate expression of tenascin-C, and define a promoter sequence that is critical for its induction. On native collagen, A10 smooth muscle cells adopt a stellate morphology and produce low levels of tenascin-C mRNA and protein, whereas on denatured collagen they spread extensively and produce high levels of tenascin-C mRNA and protein, which is incorporated into an elaborate extracellular matrix. Increased tenascin-C synthesis on denatured collagen is associated with elevated protein tyrosine phosphorylation, including activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). β3 integrin function-blocking antibodies attenuate ERK1/2 activation and tenascin-C protein synthesis. Consistent with these findings, treatment with the specific MEK inhibitor, PD 98059, results in suppression of tenascin-C protein synthesis. To investigate whether β3 integrin-dependent activation of ERK1/2 regulates the tenascin-C promoter, we transfected A10 cells with a full-length (approx. 4 kb) mouse tenascin-C gene promoter-chloramphenicol acetyltransferse reporter construct and showed that, relative to native collagen, its activity is increased on denatured collagen. Next, to identify regions of the promoter involved, we examined a series of tenascin-C promoter constructs with 5′ deletions and showed that denatured collagen-dependent promoter activity was retained by a 122-base pair element, located −43 to −165 bp upstream of the RNA start site. Activation of this element was suppressed either by blocking β3 integrins, or by preventing ERK1/2 activation. These observations demonstrate that smooth muscle cell binding to β3 integrins activates the mitogen activated protein kinase pathway, which is required for the induction of tenascin-C gene expression via a potential extracellular matrix response element in the tenascin-C gene promoter. Our data suggest a mechanism by which remodeling of type I collagen modulates tenascin-C gene expression via a β3 integrin-mediated signaling pathway, and as such represents a paradigm for vascular development and disease whereby smooth muscle cells respond to perturbations in extracellular matrix composition by altering their phenotype and patterns of gene expression.

Key words: Type I collagen; β3 integrin, MAPK; Tenascin-C promoter; Smooth muscle cell

INTRODUCTION

Abnormal accumulation of extracellular matrix (ECM) proteins in conjunction with vascular smooth muscle cell (SMC) proliferation are hallmarks of vascular disease processes including atherosclerosis, restenosis and pulmonary hypertension (Assoian and Marcantonio, 1996; Preissner et al., 1997; Jones et al., 1997a). In normal adult blood vessels, different ECM proteins interact with vascular SMC through a variety of specific cell surface receptors, including integrins (Thyberg, 1996), which play a critical role in maintaining the majority of SMC in a growth-arrested and non-motile state. In contrast, following endothelial cell injury, induction of ECM-degrading proteinases perturbs normal cell-cell and cell-ECM

interactions, leading to the activation of alternative integrin signaling pathways, and the onset of SMC growth and migration (Rabinovitch, 1997). Although recent studies have shown that integrins play a direct role in regulating SMC growth, migration, differentiation and apoptosis (Tannenbaum et al., 1995; Jones et al., 1997b), the molecular mechanisms by which integrins may modulate vascular SMC ECM gene expression during vascular remodeling have not been elucidated.

Tenascin-C (TN-C) is an ECM protein with a highly restricted pattern of gene expression, but it is prominently expressed in embryonic and adult tissues that are actively remodeling (Mackie, 1997). A variety of factors including basic fibroblast growth factor, transforming growth factor-β, interleukin-1 and -4, tumor necrosis factor-α, angiotensin II and mechanical stress have been shown to regulate TN-C expression in a cell- and tissue-type specific manner (Chiquet-Ehrismann et al., 1995). In addition, a number of studies indicate that matrix metalloproteinases (MMPs) and β3 integrins may also regulate TN-C expression. For example, following arterial injury, TN-C (Hedin et al., 1991), MMPs (Zempo et al., 1994) and β3 integrins (Slepian et al., 1998; Stouffer et al., 1998) are upregulated during the development of occlusive neointimal lesions, whereas blockade of β3 integrin binding sites (Srivasta et al., 1997) or inhibition of MMP activity (Strauss et al., 1996; Zempo et al., 1996) inhibits this process. Also, both MMP-2 (Brooks et al., 1996) and TN-C (Prieto et al., 1993; Sriramarao et al., 1993) are able to bind the αvβ3 integrin receptor, further indicating that their regulation and growth-related functions may be interdependent. In fact, we have recently shown that denaturing of native type I collagen by matrix metalloproteinases leads to SMC β3 integrin binding, which in turn activates TN-C protein synthesis (Jones et al., 1997b). However, the nature of the β3 integrin-dependent intracellular signaling molecules, and the way in which they may regulate TN-C gene expression, remain unknown.

One common response to ECM-integrin binding is activation of the mitogen activated protein kinase (MAPK) pathway (Schlaepfer et al., 1994; Chen et al., 1994; Zhu and Assoian, 1995; Maniero et al., 1997; Yokosaki et al., 1996). Family members that are sequentially activated following engagement of integrin or growth factor receptors, include the MAP kinase kinases, MEK1 and MEK2, and the extracellular regulated kinases, ERK1 (p44) and ERK2 (p42). MEK-dependent phosphorylation of ERK1 and ERK2 results in their translocation to the nucleus where they regulate a number of transcription factors involved in cell growth and differentiation (Hill and Treisman, 1995). For example, αvβ3 and α9β1 integrins activate MAPKs and support fibroblast proliferation on tenascin-C (Yokasaki et al., 1996), whereas adhesion to fibronectin or laminin activates ERK and stimulates cell proliferation via α5β1 or α6β4 integrins respectively (Chen et al., 1994; Maniero et al., 1997). Similarly, sustained activation of the MAPK pathway in endothelial cells is dependent upon adhesion and spreading via the αvβ3 integrin receptor (Elicieri et al., 1998). In addition, we have recently demonstrated that induction of an SMC endogenous vascular elastase by the transcription factor AML-1 is dependent upon both integrins and ERK1/2 activation (Thompson et al., 1996; Wigle et al., 1998).

Previous studies examining cis-regulatory elements within the full-length (approx. 4 kb) mouse TN-C promoter indicate that in fibroblasts the proximal promoter sequence, 250 bp upstream of the RNA start site, accounts for the majority of TN-C promoter activity. The specific sequences involved are highly conserved among the vertebrate TN-C proximal promoters (Copertino et al., 1995) and contain binding sites for Krox, nuclear factor 1, homeodomain proteins and a unique tenascin control element (Copertino et al., 1997). Whether these, or other cis elements within the TN-C gene promoter, are activated or suppressed in response to integrin-dependent stimulation of the MAPK pathway has not been addressed.

In this study, we have investigated the relationships between β3 integrins, ERK1/2 and TN-C expression. Using the A10 SMC line as a model system, we first show that, relative to native collagen, denatured collagen promotes extensive SMC spreading and high levels of TN-C mRNA and protein expression. Using a function-blocking anti-β3 integrin antibody and a specific MEK inhibitor, PD 98059, we demonstrate that β3 integrin-dependent activation of ERK1 and ERK2 leads to increased TN-C expression. Next, to investigate whether β3 integrin-dependent activation of ERK1/2 regulates the TN-C promoter, we transfected A10 cells with a full-length (approx. 4 kb) mouse TN-C gene promoter-CAT reporter construct and showed that, relative to native collagen, its activity is increased on denatured collagen. Using a series of TN-C promoter constructs with 5′ deletions and a mutation in an NF-1 binding site, we showed that denatured collagen-dependent promoter activity was retained by a 122-bp element, located −43 to −165 bp upstream of the RNA start site. Activation of this element is suppressed either by blocking β3 integrins, or by preventing ERK1/2 activation. These observations demonstrate that SMC binding to β3 integrins activates the MAPK pathway, which is required for the induction of TN-C gene expression via a potential ECM response element in the TN-C gene promoter. Our data provide a mechanism by which remodeling of type I collagen modulates TN-C gene expression via a β3 integrin-mediated signaling pathway, and represents a paradigm for vascular development and disease whereby SMCs respond to perturbations in ECM composition by altering their phenotype and patterns of gene expression.

MATERIALS AND METHODS

Cell culture

A10 vascular SMCs, a fetal rat thoracic aorta cell line (American Tissue Culture Collection, VA), were routinely maintained in Medium 199 (M199) containing 10% heat-inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY), 10 units/ml penicillin G sodium, 10 mg/ml streptomycin sulfate, 0.25 mg/ml amphotericin B and 0.1 mg/ml gentamicin sulfate (Gibco-BRL). Cells were passaged by trypsinization using 0.05% trypsin/EDTA (Gibco-BRL). Type I collagen substrates were prepared based on methods that have been described previously (Jones et al., 1997b). Briefly, for native type I collagen, 0.8 ml of a 3.1 mg/ml solution of bovine dermal type I collagen (Vitrogen 100, Collagen Corp., CA), 0.1 ml of 0.1 M NaOH and 0.1 ml of 10× PBS were mixed at 4°C for a final collagen concentration of 2.48 mg/ml. Fibrillogenesis was initiated overnight in a humid 5% CO_2 environment at 37°C. For assessment of the effects of heat-denatured collagen, cells were plated on 2.48 mg/ml of type I collagen that had been boiled for

60 minutes with 0.02 M acetic acid, neutralized with 0.1 M NaOH, and then air-dried to the bottom of each dish. Before plating cells, native or denatured collagen substrates were rinsed extensively (3× 3 hours) with serum-free M199. Cells were plated at a density of 1.76×10^4 cells/cm^2 on collagen substrates in M199 plus 2% FCS. 6 hours after plating, cells were rinsed three times with serum-free medium, and then with M199 plus 0.1% bovine serum albumin (Boehringer-Mannheim; Fraction V) and 0.1% FCS. Cultures were maintained for an additional 48 hours in M199 plus 0.1%BSA/FCS. For β3 integrin blocking studies, cultures were supplemented 24 hours after plating with 25 μg/ml of anti-rat β3 integrin IgG (Pharmingen; CD61), or an equivalent concentration of control IgG (Dako). For MEK inhbition, cultures were supplemented 24 hours after plating with 50 μm PD 98059 (2′-amine-3′-methoxyflavone, Calbiochem; Dudley et al., 1995), or with DMSO (vehicle) as a control. All experiments were performed in triplicate, with the exception of northern analysis which was performed in duplicate.

N rthern analysis for tenascin-C

To determine whether denatured collagen upregulates TN-C at the mRNA level, we carried out northern blot analysis. To extract total RNA, A10 SMCs cultured on native or denatured type I collagen were treated with 3 ml of boiling hot RNAzol (Cinna/Biotex) per 10 cm diameter dish. Cells were scraped and transferred to a 15 ml tube and mixed with 1/10 volume of chloroform and centrifuged at 12,000 *g* for 15 minutes at 4°C. The aqueous phase was mixed with an equal volume of isopropanol before precipitation overnight at −20°C. Samples were centrifuged at 12,000 *g* for 15 minutes at 4°C, and the resulting RNA pellets were washed twice in 75% ethanol and resuspended in 0.1%SDS in DEPC-water. A 10 μg sample of total RNA per lane was electrophoresed on a 1% agarose/formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham Life Sciences Inc) by capillary transfer for 12 hours, and then cross-linked by exposure to a UV source. Hybridizations were performed at 68°C with a ^{32}P-labelled random-primed probe prepared from a 250-bp cDNA sequence derived from the seventh fibronectin type III universal domain of rat TN-C in QuikHyb solution. The relative amount of TN-C mRNA detected in each sample was analyzed by densitometry and corrected for loading with 28S rRNA measurements detected after ethidium bromide staining of agarose/formaldehyde gels. All experiments were performed in duplicate.

Western immunoblot analysis for tenascin-C, tyrosine-phosphorylated proteins and activated forms of ERK1 and ERK2

Expression of TN-C and detection of activated tyrosine-phosphorylated proteins, including ERK1 and ERK2, were determined by immunoblotting equal numbers (2.25×10^5 cells) of whole cell lysates with appropriate specific antisera or antibodies, using western analysis. Briefly, cells cultured either on native or denatured type I collagen substrates were harvested in ice-cold PBS buffer (pH 7.4) containing 1 mM benzamidine, 1 mM PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM NaF, 2 mM Na_3VO_4 and 1 mM $MgCl_2$. An equal volume of SDS-PAGE sample buffer containing 0.06% Bromophenol Blue and 2% β-mercaptoethanol was added to each sample before boiling for 5 minutes. Samples were then passed through a 26-gauge needle and centrifuged before loading and electrophoresis on 4%-12% polyacrylamide gels (Novex). In order to confirm that equal amounts of protein were loaded, duplicate SDS-PAGE gels were run in parallel with experimental gels and then stained with Coomassie Blue. Proteins were transferred to Immobilon polyvinyl fluoride membranes (Millipore Corp), and these were blocked for 1 hour at 37°C in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween-20), supplemented with 0.1% BSA. To detect TN-C protein, membranes were sequentially incubated with a rabbit polyclonal anti-TN-C antiserum (a gift from Harold Erickson, diluted 1:500; 0.2 μg/ml), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Gibco-BRL) diluted 1/5000 (0.1 μg/ml) in wash buffer. To detect tyrosine-phosphorylated proteins, blots were incubated for 30 minutes with horse rabbit peroxidase-conjugated anti-phosphotyrosine antibody, diluted 1/2500 (0.1 μg/ml) in wash buffer. To detect phosphorylated ERK1 and ERK2, we used a rabbit polyclonal IgG (PhosphoPlus, NEB; 0.1 μg/ml), followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Thereafter, all membranes were rinsed in wash buffer for 3× 15 minutes and TN-C and tyrosine-phosphorylated proteins, including ERK1 and ERK2, were visualized by enhanced chemiluminescence (Amersham ECL kit), before exposure to Kodak X-Omat film.

Immunofluorescence detection of tenascin-C and CAT protein

For immunofluorescence studies, SMCs were cultured on glass coverslips (12 mm, No. 1 thickness) coated with 2.48 mg/ml of neutralized native or denatured type I collagen. For detection of TN-C and CAT protein, cells were fixed in cold 100% methanol for 30 minutes at −20°C. After preincubation in blocking solution containing 10% normal goat serum and 2% BSA for 1 hour at room temperature, slides were incubated overnight at 4°C with either a rabbit polyclonal anti-TN-C antiserum, or a rabbit polyclonal anti-CAT IgG (5 Prime-3 Prime Inc, Boulder, CO), diluted 1:100 in PBS with 1% BSA. Cells were washed in PBS, and incubated for 1 hour at room temperature with a 1:50 dilution of rhodamine-conjugated goat anti-rabbit antibody. For the CAT protein experiments, nuclei were stained with 4′-6-diamidino-2-ohenylindole (DAPI), diluted 1:10000 in PBS. All coverslips were mounted onto glass slides using Antifade reagent (Molecular Probes). Observations and photomicrographs were obtained with an Olympus fluorescence microscope using epifluorescence.

Immunoprecipitation of tenascin-C protein

For immunoprecipitation of radiolabelled TN-C protein, SMCs were cultured on collagen gels in 35 mm diameter tissue culture dishes for 48 hours, and then incubated in cysteine/methionine-free medium (ICN) supplemented with 0.1% BSA for 3 hours, and then pulse-labelled with 100 mCi/ml [^{35}S]methionine/cysteine Translabel (ICN) for 21 hours. Cells were collected in RIPA buffer and cleared by centrifugation. Total cpm were normalized by TCA precipitation and equal cpm (5×10^5 cpm/sample) were immunoprecipitated with 5 μl rabbit polyclonal antisera raised against TN-C (Erickson). Immune complexes were precipitated with 25 μl Protein A-Sepharose (Sigma), washed in 1× RIPA buffer, 50 mM Tris, pH 7.4, and resuspended in 40 μl 2× SDS-PAGE sample buffer. Proteins were separated on 6% SDS/polyacrylamide gels, fixed, dried and exposed to Kodak X-Omat film for 2 days.

Tenascin-C promoter CAT reporter plasmids

The 5′ deletions in the mouse TN-C promoter were generated by PCR using the 4.2 kb TN-C genomic fragment as a template, and fragments were inserted into the promoterless vector pBasic as previously described (Copertino et al., 1995, 1997). All constructs including TN12M, which contains a 2-base pair substitution in the NF-1 site, are illustrated and described in Fig. 3.

Analysis of tenascin-C promoter-CAT reporter constructs in A10 cells

Cells were cultivated overnight on native or denatured type I collagen in M199 plus 2% FCS. 12 hours after plating, the medium was removed, and replaced with M199 with 0.1%FCS and 0.1% BSA. 33 hours after plating, cultures were rinsed for 3× 1 hour with antibiotic- and serum-free M199. For each 60 mm diameter tissue culture dish, 2.5 μg TN-C-CAT reporter plasmid DNA was co-transfected with 0.5 μg β-galactosidase plasmid DNA (pSV-β-gal; Promega) using Lipofectamine reagent (Gibco-BRL). 5 hours after transfection, M199 was removed and replaced with M199 plus 0.1% BSA. For anti-β3

integrin antibody blocking studies, medium was supplemented with 25 μg/ml anti-rat β3 integrin IgG (Pharmingen; CD61), or an equivalent concentration of control IgG (Dako). For MEK inhibition studies, cultures were supplemented 24 hours after plating with 50 μm PD 98059 (2′-amine-3′-methoxyflavone; Calbiochem; Dudley et al., 1995), or with DMSO (vehicle) as a control. 72 hours after plating, transfected cells were rinsed 3 times in warm PBS, pH 7.4, and then scraped into 1.5 ml of ice-cold PBS. Cell pellets were rinsed twice in cold PBS, the supernatant removed, and pellets frozen at −20°C overnight in 80 μl of 0.25 M Tris buffer, pH 7.8. To lyse cells, pellets were freeze-thawed three times, centrifuged, and the supernatant transferred to a fresh tube. β-galactosidase assays were carried out using standard protocols to normalize for transfection efficiency (Zhou et al., 1997). This was achieved by mixing 15 μl of cell extract with 15 μl of 0.25 M Tris, 3 μl 100× Mg solution, 66 μl 1× ONPG (o-nitrophenyl-β-D-galctopyranoside) and 201 μl of 0.1 M sodium phosphate. Reactions were incubated at 37°C, and then terminated with 0.5 ml of 1 M Na_2CO_3 before measuring the optical density at a wavelength of 420 nm. Cell lysates containing equal amounts of β-galactosidase activity were used for CAT assays with [^{14}C]chloramphenicol (ICN) and acetyl-CoA. The acetylated products were chromatographed on thin-layer plates in 95% chloroform/5% methanol. The CAT activity was assessed by densitometry using the Biorad GelDoc system and NIH image software.

Statistical analyses

All statistical assessments were compared by one-way analysis of variance and Student-Newman-Keuls post hoc analysis. $P<0.05$ was considered statistically significant. Values are means ± s.e.m.

RESULTS

Denatured type I collagen upregulates tenascin-C mRNA and protein in rat A10 vascular smooth muscle cells

We have previously shown that TN-C protein expression in primary pulmonary artery SMC is upregulated by proteolyzed and/or heat-denatured type I collagen, relative to the native form of this protein (Jones et al., 1997b). In the present study, we used the A10 fetal rat aortic SMC line as a model system to further investigate how denatured collagen upregulates TN-C. On native collagen, A10 SMC adopted a stellate morphology (Fig. 1A), and expressed low steady-state levels of a 6.5 kb TN-C mRNA as determined by northern blot analysis. In contrast, on denatured collagen, A10 cells spread extensively, and showed a substantial increase in TN-C mRNA (Fig. 1B). This increase in TN-C mRNA was reflected by a significant increase in TN-C protein expression as determined by western immunoblotting ($P<0.05$) (Fig. 1C). In addition, immunofluorescence detection of TN-C protein in cells cultured on either native or denatured collagen revealed that TN-C protein is deposited in an elaborate ECM on denatured collagen (Fig. 1D). These results establish that cell morphology and TN-C expression in A10 cells are similar to primary SMC cultured on native and denatured type I collagen, and show that increased mRNA levels of TN-C on denatured type I collagen are reflected by increased expression and deposition of TN-C protein.

Upregulation of tenascin-C synthesis on denatured collagen is dependent upon β3 integrins and activation of ERK1/2

Integrin-dependent regulation of gene expression and cell behaviour relies upon tyrosine phosphorylation of a number of key intracellular proteins such as focal adhesion kinase (FAK) and the mitogen activated protein (MAP) kinases (reviewed in

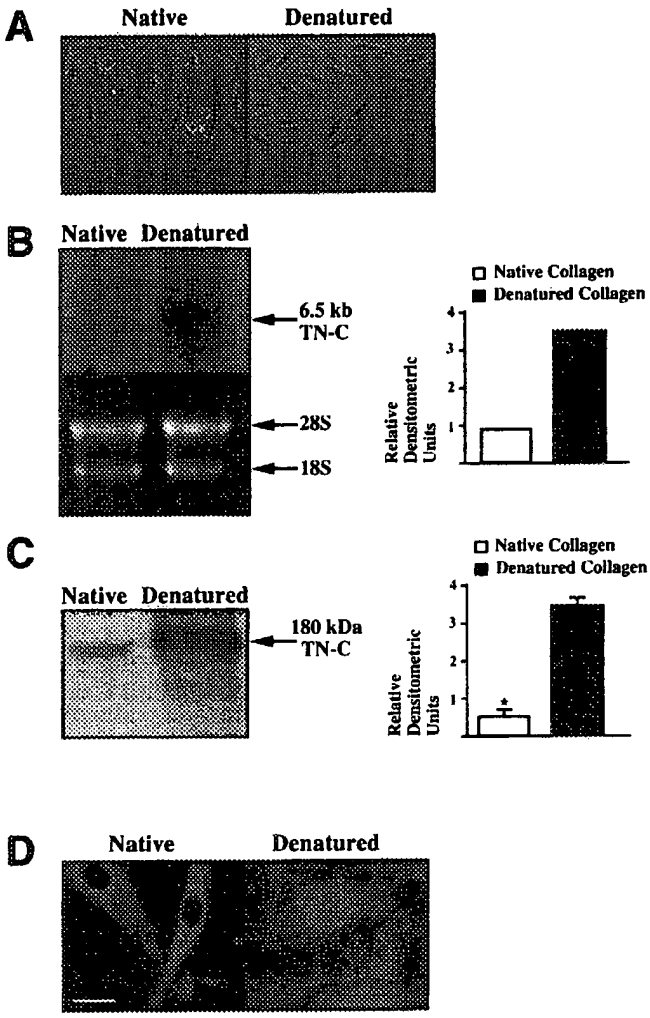

Fig. 1. Effect of denatured type I collagen on smooth muscle cell morphology and TN-C production. (A) Representative phase-contrast photomicrographs showing A10 SMC cultured on native or denatured type I collagen in serum-free medium (SFM). Cell spreading is enhanced on denatured collagen. (B) Representative northern blot for TN-C mRNA in A10 cells cultured in SFM on native or denatured collagen. Densitometric analysis, from two separate experiments, of the 6.5 kb TN-C isoform from autoradiograms normalized to 28S rRNA loading control shows that steady-state levels of TN-C mRNA levels are increased approximately 3.5-fold on denatured versus native type I collagen. (C) Representative western immunoblot for TN-C protein from A10 cells cultured on native or denatured type I collagen in SFM. Densitometric analyses of three separate experiments show a significant 3.5-fold increase in TN-C on denatured collagen. Values are means ± s.e.m. (*$P<0.05$). (D) Representative immunofluorescence photomicrographs showing the distribution of TN-C protein in A10 cells cultured in SFM on native or denatured type I collagen. Low levels of cell-associated TN-C are evident in cultures on native type I collagen, whereas high levels of extracellular TN-C are apparent on denatured collagen. Bars, 40 μm (A); 10 μm (D).

Juliano et al., 1996). However, the ability of integrins and MAP kinase mediators, including MEK and ERK1/2, to modulate ECM gene expression has not been explored. Since we have already shown that primary SMCs use β3 integrins to support both basal and induced levels of TN-C protein synthesis on native and denatured type I collagen substrates, respectively (Jones et al., 1997b), we were interested in determining whether A10 SMC β3 integrins interact with denatured collagen to promote TN-C expression via the MAP kinase signaling pathway. Western immunoblot analysis of cell lysates demonstrated that, relative to native collagen, culturing A10 cells on denatured collagen increased the levels of tyrosine-phosphorylated proteins, including those of approximate molecular mass 180, 125, 100, 60 and 40 kDa (Fig. 2A, left). Treatment of A10 cells cultured on denatured collagen with a function-blocking anti-β3 integrin monoclonal antibody attenuated the majority of tyrosine phosphorylation activity, including proteins of about 40 kDa molecular mass (Fig. 2A, center). Incubation with the specific MEK inhibitor, PD 98059, resulted in a more selective inhibition of these proteins, which likely represent the MEK substrates, ERK1 (44 kDa) and ERK2 (42 kDa) (Fig. 2A, right). Western immunoblot analyses of A10 cells cultured on native and denatured collagen, using antibodies that recognize the phosphorylated (activated) forms of ERK1 and 2, established that activation of these proteins was significantly increased on denatured collagen (Fig. 2B, left), whereas treatment with the anti-β3 integrin function blocking antibody (Fig. 2B, center) or MEK inhibitor (PD 98059) (Fig. 2B, right) on denatured collagen resulted in a significant loss of ERK1/2 activity. To determine whether denatured collagen, β3 integrins and ERK1/2 regulate TN-C protein synthesis, we repeated the studies described above, and immunoprecipitated TN-C protein using metabolically labeled A10 cell lysates. Tenascin-C protein synthesis was significantly increased on denatured collagen (Fig. 2C, left), but was suppressed following β3 integrin blockade (Fig. 2C, center), or following inhibition of MEK activation with PD 98059 (Fig. 2C, right). These data indicate that on denatured collagen, β3 integrin-dependent activation of ERK1 and ERK2 regulates TN-C synthesis in vascular SMCs.

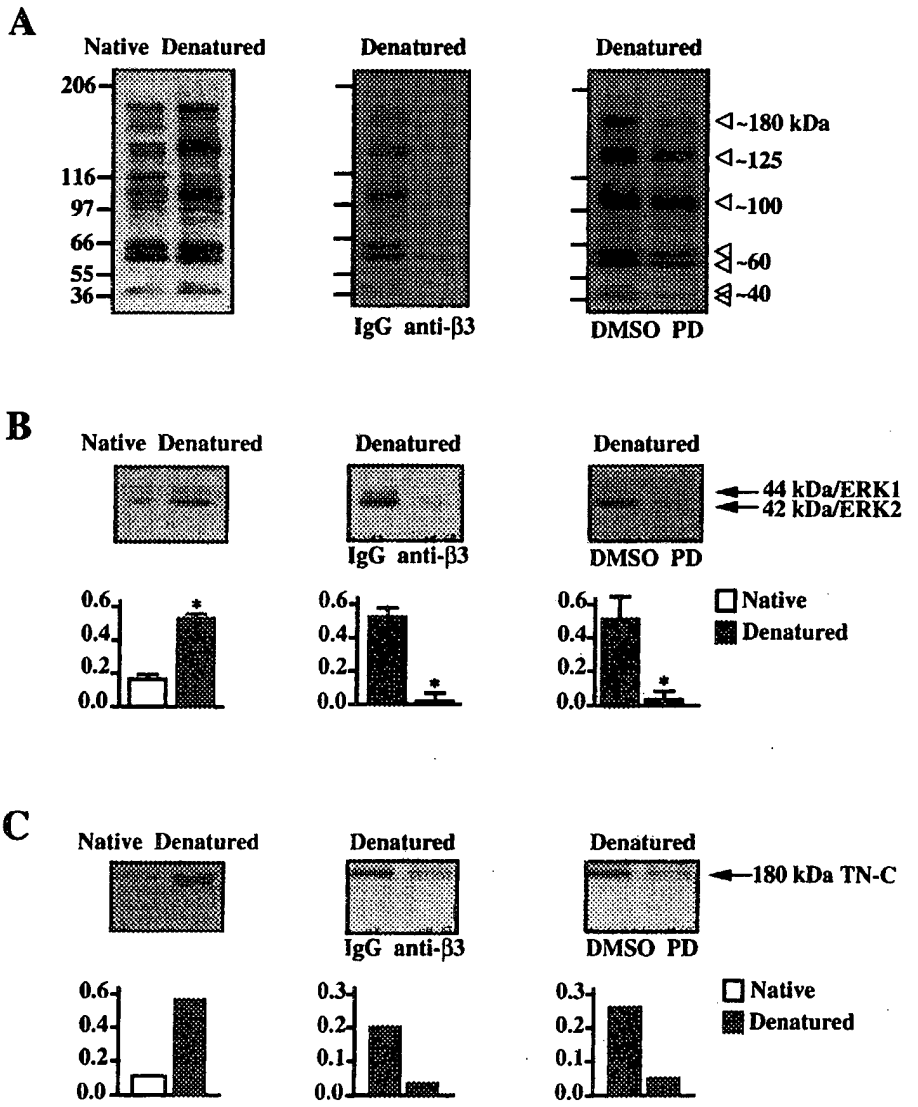

Fig. 2. β3 integrin activation of ERK1/2 and TN-C protein synthesis in SMCs cultured on denatured type I collagen. (A) Representative western immunoblots show that SMC culture on denatured type I collagen results in increased tyrosine phosphorylation, including a number of prominent proteins denoted by open arrowheads (left). The β3 integrin blocking antibody represses this increase in tyrosine phosphorylation on denatured collagen, whereas contol IgG has no effect (center). The MEK kinase inhibitor (PD 98059; PD) more selectively suppresses proteins of about 40 kDa (right). (B) Representative western immunoblots and relative densitometric analyses of three separate experiments to determine differences in activation (phosphorylation) status of ERK1 and ERK2 on native versus denatured type I collagen (left), and on denatured collagen following β3 integrin blockade (center), or following MEK inhibition with PD 98059 (PD) (right). Values are means ± s.e.m. (*P<0.05). (C) Representative autoradiograph of two TN-C immunoprecipitation experiments with similar results and relative densitometric analyses from all experiments under the conditions shown in B.

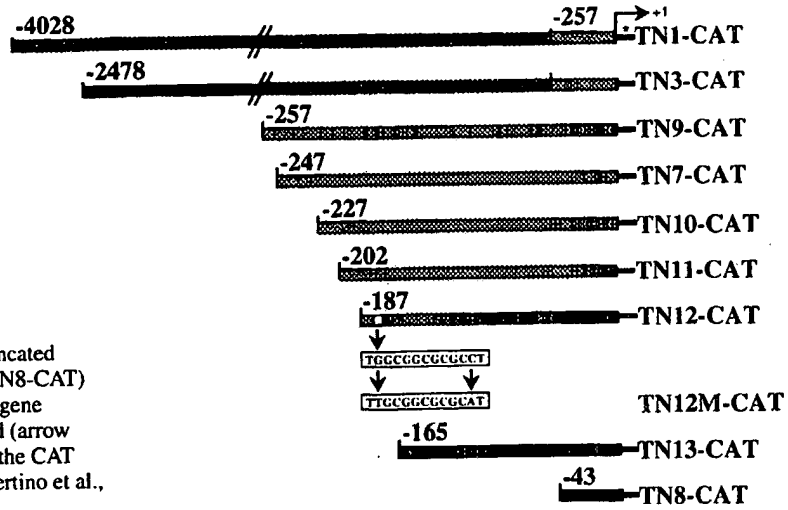

Fig. 3. Schematic structure of full-length (TN1-CAT), truncated (TN3-, TN9-, TN7-, TN10-, TN11-, TN12-, TN13- and TN8-CAT) and mutated (TN12M-CAT) mouse TN-C promoter/CAT gene reporter constructs. The transcription start site is indicated (arrow labeled +1) and a 147-bp sequence (*) at the 3′ border of the CAT constructs relative to the transcription initiation site (Copertino et al., 1995, 1997).

Activation of the tenascin-C gene promoter by denatured collagen requires a 122-base pair cis lement

To establish whether upregulation of TN-C mRNA expression by denatured type I collagen was associated with increased transcription, we used a full-length 4028 bp mouse TN-C promoter linked to a CAT reporter gene, designated TN1-CAT, and examined CAT protein accumulation and activity in A10 cells following transient transfection (Fig. 3) (Copertino et al., 1995; 1996). As a control, we transfected cells with the pCATbasic parent plasmid vector. Immunofluorescence staining for CAT protein in TN1-CAT transfected cells on native collagen revealed low levels of CAT protein accumulation, whereas on denatured collagen, high levels of CAT expression were observed (Fig. 4). No CAT immunofluorescence staining was detected in cells transfected with the pCATbasic promoterless vector (data not shown).

Direct assessments of TN1-CAT activity on native verus denatured collagen also demonstrated that transcriptional activation of the mouse TN-C promoter in cells cultured on native collagen produced levels comparable to the parent pCAT basic plasmid (Fig. 5A), whereas denatured collagen significantly increased TN1-CAT activity (Fig. 5A). To estabish which regions of the TN-C gene promoter were responsive to denatured collagen, we transfected A10 cells with TN-C promoter-CAT constructs which had been successively deleted from the 5′ end of the promoter, as well as a 2-base substitution in the NF-1 site of TN12-CAT, which is known to prevent NF-1 binding (TN12M) (Fig. 3; Copertino et al., 1997). Deletion of a 1550-bp sequence between positions –4028 and –2478 bp (TN3-CAT) resulted in a significant loss of CAT activity to pCAT basic levels (Fig. 5B). Deletion of an additional 2221 bp between positions –2478 and –257 (TN9-CAT) significantly restored CAT activity to the levels previously observed for TN1-CAT (Fig. 5B). Successive deletions from –257 to –165 (TN9-, TN7-, TN10-, TN11-, TN12- and TN13-CAT), as well as TN12M, had no significant effect on CAT activity compared to TN1-CAT (Fig. 5B). Deletion of the remaining 122 bp, however, resulted in a significant tenfold reduction in CAT activity that was comparable to pCAT basic transfectants (Fig. 5B), and to A10 cells transfected on native collagen with these same constructs (data not shown). These data indicate that the 122-bp sequence present in TN13-CAT, but absent in TN8-CAT, is essential for the positive activity of the TN-C promoter induced by denatured collagen.

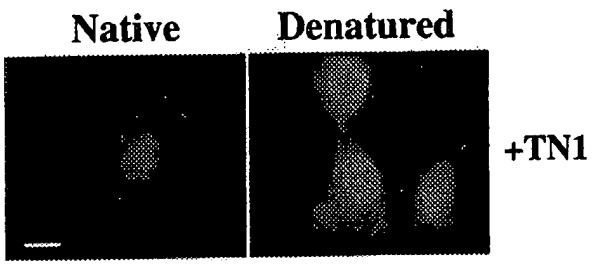

Fig. 4. Denatured type I collagen activates the full-length mouse TN-C gene promoter in transiently transfected A10 SMC. Representative immunofluorescence staining for CAT protein in A10 cells maintained in SFM on either native or denatured type I collagen following transient transfection with the full-length mouse TN-C promoter-CAT construct, TN1-CAT. Cytoplasmic accumulation of CAT protein (red) is markedly increased on denatured collagen. Bar, 15 μm.

Activation of the 122-base pair tenascin promoter element is dependent upon β3 integrins and MAP kinases

To determine whether activation of the 122-bp denatured collagen-responsive element was also dependent upon β3 integrin binding and ERK1/2 activation, we transfected A10 cells on denatured collagen with TN-CAT constructs and assessed CAT activity following either β3 integrin blockade with the function blocking antibody, or following inhibition of MEK activity with PD 98059. Compared to cultures exposed to control IgG, treatment with anti-β3 integrin antibodies significantly reduced the TN1- and TN13-CAT activity to TN8-CAT basal levels (Fig. 6A). Inhibition of ERK1/2 activation

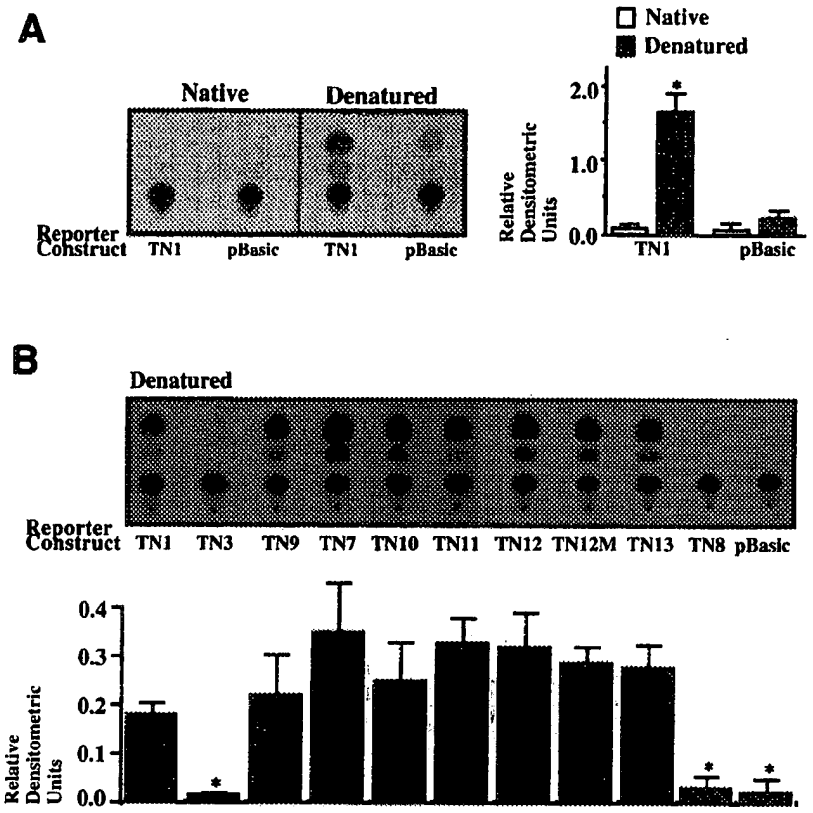

Fig. 5. Minimum promoter requirement for TN-C promoter-driven CAT gene expression in A10 SMC transiently transfected with deletion constructs and cultivated on denatured collagen. (A) Representative CAT activity in A10 SMC following transient transfection with TN-1 and pCATbasic constructs demonstrates that the TN-C gene promoter is activated by denatured collagen. The densitometric analyses from three different experiments is on the right. Values are means ± s.e.m. (*P<0.05). (B) Representative CAT activity and relative densitometric analyses from three separate experiments. Deletion of a 1550-bp sequence between positions −4028 and −2478 bp (TN3-CAT) resulted in a significant loss of CAT activity to pCAT basic levels. Deletion of an additional 2221 bp between positions −2478 and −257 (TN9-CAT) significantly restored CAT activity to the levels previously observed for TN1-CAT (Fig. 5B). Successive deletions from −257 to −165 (TN9-, TN7-, TN10-, TN11-, TN12- and TN13-CAT), as well as TN12M, had no significant effect on CAT activity compared to TN1-CAT. Deletion of the remaining 122 bp resulted in a significant tenfold reduction in CAT activity that was comparable to pCAT basic transfectants. Values are means ± s.e.m. (*P<0.05).

with PD 98059 had a similar effect (Fig. 6B). Together, these data indicate that denatured collagen promotes transcription of the TN-C gene promoter via β3 integrins and a MAP kinase signaling pathway that involves activation of ERK1 and 2.

DISCUSSION

It is firmly established that the ECM and integrins provide cells with critical cues that regulate proliferation, migration, differentiation and apoptosis. While ECM and integrin-dependent activation of MAPKs has been implicated in both transcriptional and non-transcriptional regulation of cell behavior (Hill and Treisman, 1995; Klemke et al., 1997; Elicieri et al., 1998), no study has detailed how integrin-dependent activation of MAPKs may regulate ECM gene expression. In the present work, we used function-blocking integrin antibodies and a synthetic MEK inhibitor to show that

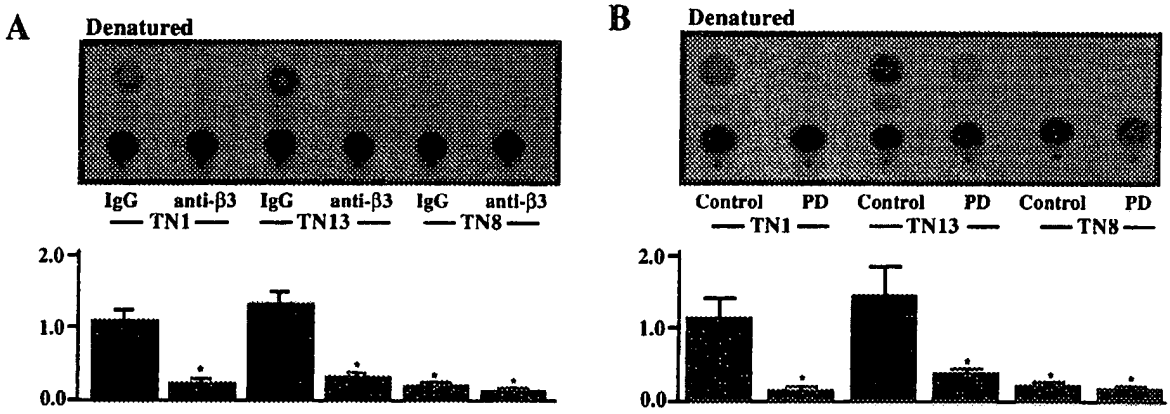

Fig. 6. Activation of a β3 integrin-MAP kinase-dependent ECM response element in the TN-C promoter. Representative CAT assays and densitometric analyses from three separate experiments. (A) CAT assays following transient co-transfection of TN1-, TN13- and TN8-CAT constructs shows loss of CAT activity in the presence of the β3 integrin blocking antibody with TN1 or TN13 to the level observed with TN 8, which lacks the putative 122-bp ECM response element. Values are means ± s.e.m. (*P<0.05). (B) CAT assays, as in A and showing similar results using the MEK inhibitor PD 98059 (PD). Values are means ± s.e.m. (*P<0.05).

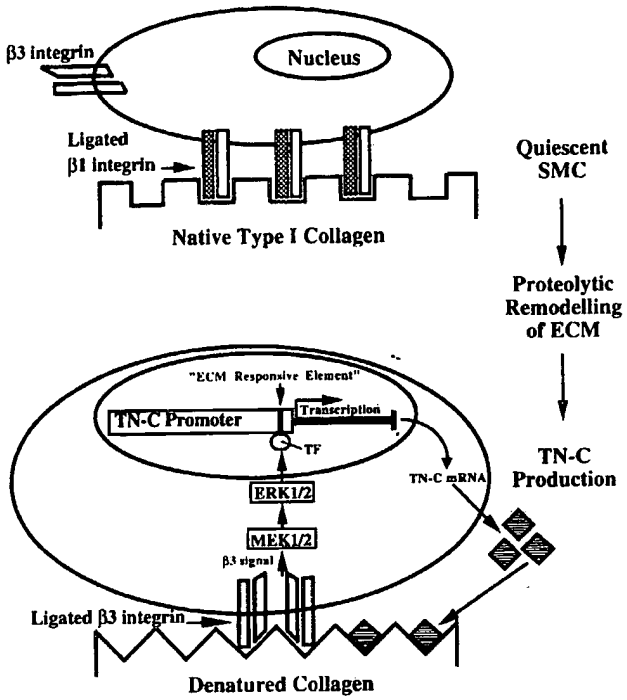

Fig. 7. Hypothetical model showing how denatured type I collagen may drive vascular smooth muscle cell tenascin-C gene expression in a manner that is dependent upon a β3 integrin-mediated mitogen-activated protein kinase pathway and a 122-bp promoter element. On native type I collagen, SMCs attach and spread using β1 integrins. Under serum-free conditions, these cells become quiescent. Degradation of type I collagen leads to exposure of cryptic RGD sites in type I collagen that preferentially bind and activate β3 integrins which, in turn, activate the MAPKs, ERK1 and ERK2. These factors target transcription factors which bind to and activate a 122-bp ECM responsive element in the TN-C gene promoter, leading to transcription and increased deposition of TN-C protein.

β3 integrin binding to denatured type I collagen activates ERK1/2 and ultimately TN-C promoter activity and gene transcription. In addition, we used a series of truncated and mutated TN-C gene promoter-reporter constructs to localize a 122-bp element within the TN-C promoter that is responsive to these β3 integrin and ERK1/2-derived signals. The present study therefore provides new evidence to show how remodeling of type I collagen in blood vessels may influence integrin signaling functions, and ultimately patterns of ECM gene expression. Based on our findings, we propose a hypothetical model for the transcriptional regulation of the TN-C gene by β3 integrins and ERK1/2 during normal and pathological remodeling of the vasculature (Fig. 7).

Normal vascular SMCs are surrounded by a complex interconnecting network of ECM proteins including type I collagen, which interacts with the α2β1 integrin receptor (Messent et al., 1998). During the development and progression of occlusive arterial disease, however, the composition of the vascular ECM is modified and SMCs adopt both a proliferative and migratory phenotype (Jones and Rabinovitch, 1996; Jones et al., 1997b; Zhou et al., 1997). Matrix metalloproteinases are a class of zinc-dependent enzymes which are upregulated and activated in remodeling vascular tissues (Bendeck et al., 1994; Zempo et al., 1994; Strauss et al., 1996), where they alter cell-ECM interactions and integrin-dependent intracellular signal transduction pathways (Basbaum and Werb, 1996). A number of studies have shown that expression of β3 integrins and TN-C also increases with the development of neointimal lesions (Stouffer et al., 1998; Slepian et al., 1998; Jones et al., 1997a; Zempo et al., 1994; Bendeck et al., 1993). Furthermore, we have shown that MMPs upregulate TN-C protein synthesis, and that TN-C functions to support SMC proliferation by promoting epidermal growth factor receptor clustering on SMC surfaces (Jones et al., 1997b). Mechanistically, MMP- or heat-denaturation of type I collagen exposes cryptic RGD ligands that preferentially bind αvβ3 integrins rather than α2β1 integrins to promote cell shape changes and survival (Montgomery et al., 1994). Similarly, a switch in integrin ligation from α2 to αv or a5β1 integrins occurs when the triple helical configuration of type I collagen is partially degraded or lost (Davis, 1992; Tuckwell et al., 1994; Messent et al., 1998). For example, attachment of fibroblasts to native or a native 3/4 fragment of type I collagen via the whole α2β1 integrin or the α2-domain is lost when the triple helical structure of this substrate is destroyed by heating (Messent et al., 1998). In addition, our previous studies have shown that inhibition of type I collagen remodeling with a specific MMP inhibitor suppresses basal levels of TN-C expression, whereas denaturing type I collagen supports TN-C protein synthesis through β3 integrin ligation (Jones et al., 1997b). The present results extend these findings by providing novel functional data which show that β3 integrin binding to denatured collagen activates ERK1 and 2, which in turn regulate TN-C gene transcription. Interestingly, Koyama et al. (1996) have also shown that alterations in type I collagen structure from a native fibrillar to a denatured monomer form promotes arterial SMC spreading and cell growth, but this was dependent upon α2β1 integrins. Given the wealth of information which indicates that denatured collagen is unable to bind β1 integrins, and that β3 integrins are preferentially expressed in remodeling vascular tissues in vivo, it is plausible that these α2β1 integrin-dependent events may be mediated by other β1 integrin ligands, such as TN-C (Sriramarao et al., 1993), produced in response to denatured collagen and β3 integrins.

Considerable evidence demonstrates that cell shape and three-dimensional tissue organization, orchestrated by the ECM and integrins, have a profound influence on gene expression and cell behavior (Folkman and Moscona, 1978; Mooney et al., 1995; Weaver et al., 1997; Kheradmand et al., 1998). A number of studies indicate that expression of ECM proteins may also be regulated by the substratum (Hedin et al., 1988; Streuli and Bissell, 1990; Jones et al., 1995; 1997b). For example, fibronectin synthesis in cultured SMCs is reduced in cells grown on fibronectin compared to laminin (Hedin et al., 1988), whereas expression of fibronectin mRNA by mammary epithelial cells is suppressed on type I collagen, and increased on tissue culture plastic substrata, which promotes cell spreading (Streuli and Bissell, 1990). Consistent with this, our previous studies using a mammary epithelial cell strain indicate that cell spreading on plastic may be characterized by increased TN-C mRNA expression, whereas laminin-induced cellular rounding is accompanied by inhibition of TN-C mRNA production (Jones et al., 1995). Furthermore, SMCs rounding

on contracted or floating type I collagen gels is characterized by inhibition of TN-C production and the onset of apoptosis (Jones et al., 1997b). The present results show that ERK1/2 activity and TN-C expression and deposition are repressed in SMCs cultured on native type I collagen, where cell spreading is limited, and increased on denatured collagen, where extensive cell spreading occurs. These data further support the idea that expression and deposition of TN-C are intimately linked to changes in cell shape. Other studies have shown that cell shape affects MAPK activation. For example, while PDGF can transiently and rapidly activate ERK in fibroblasts, cell spreading induced by adhesion to fibronectin results in a more gradual and sustained activation of ERK, which is permissive for cell growth (Zhu and Assoian, 1995). Similarly, growth factor-dependent activation of ERK2 can only occur when non-transformed cells adhere to fibronectin (Renshaw et al., 1997). Therefore, it is likely that the SMC shape afforded by denatured collagen and β3 integrins contributes to sustained ERK1/2 activation and TN-C transcription.

Having established that denatured collagen regulates TN-C at the mRNA level, and that β3 integrin binding to denatured type I collagen induces TN-C via an ERK1/2-mediated pathway, our next goal was to localize the region(s) of the TN-C promoter that was responsive to β3 integrin-derived signals. Transient transfection of SMC with CAT reporter constructs under the control of the full-length 4-kb mouse TN-C promoter showed that denatured collagen induces TN-C transcription, whereas native collagen has the opposite effect. We then used TN-C promoter-reporter constructs containing proximal promoter sequences, as well as upstream flanking sequences, to show that a 2.2-kb upstream sequence, between positions −2478 and −257, represses TN-C transcription on denatured collagen. This finding is consistent with earlier studies in fibroblasts showing that this segment of the TN-C promoter reduces the activity of the proximal promoters of the chicken, mouse and human genes (Jones et al., 1990; Copertino et al., 1995; Gherzi et al., 1995), and it also indicates that this region may harbor one or more cis elements that repress the β3 integrin signaling pathway. Repression of transcription as a mechanism for regulating gene expression has also been demonstrated for other genes (Kageyama and Pastan, 1989), and further studies will be needed to produce conclusive data about the nature of the putative inhibitory sequence(s) in the mouse TN-C promoter. Recent studies, however, have shown that the human TN-C promoter contains a homeodomain binding site at position −534, which binds OTX2 to trans-repress human TN-C promoter activity (Gherzi et al., 1997). This binding site is conserved between mouse (position −560) and human TN-C promoters, and it would therefore be of interest to determine whether OTX2 has a similar effect on the activity of the mouse TN-C promoter in SMC.

The ability of the TN-C promoter to respond to β3 integrins and ERK1/2-derived signals is retained by a 122-bp sequence, located between positions −165 and −43, and deletion of this element resulted in a total loss of β3 integrin-induced transcriptional activity in transiently transfected SMCs cultured on denatured type I collagen. That is, activation of the 122-bp element by denatured collagen could be inhibited by blocking β3 integrins with specific antibodies, or by inhibiting ERK1/2 activation with PD 98059. This region of the TN-C promoter is highly conserved between species (Copertino et al., 1995), and encompasses a homopolymeric (dA) tract at position −99, which is thought to modulate access to the transcriptional machinery via its effects on DNA bending and nucleosome exclusion (Reardon et al., 1993; Nelson et al., 1987). The 122-bp sequence also harbors a potential homeodomain binding site located −57 bp upstream from the start site. Earlier studies by Jones et al. (1992) demonstrated that TN-C is a target for the homeobox-containing gene *Evx-1* which, in addition to TN-C mRNA (Hedin et al., 1991), is upregulated in vascular SMC following injury in vivo (Miano et al., 1996). Similarly, recent studies have shown that expression of *HoxB7* and *HoxC9*, is restricted to human fetal SMC whereas no expression was observed in adult SMC lines or adult aorta (Miano et al., 1996). Since TN-C (Jones and Rabinovitch, 1996; Jones et al., 1997a,b) and Hox genes have each been shown to enhance the proliferative potential of cells (Krosl et al., 1998), it is tempting to speculate that transcription factors of the Hox gene family may modulate cell growth via their effects on TN-C gene expression,

The finding that the 122-bp element is necessary for β3 integrin-dependent TN-C transcriptional activity on denatured type I collagen is in contrast to previous studies showing that deletion of this element has no significant effect on the TN-C promoter activity in NIH 3T3 cells and C6 cells cultured on tissue culture plastic (Copertino et al., 1997). In fact, deletion of this sequence resulted in a twofold increase in transcriptional activity in N2A cells (Copertino et al., 1997). Together, these data indicate that the activity of the poly(dA)/Antennapedia HD region of the TN-C is not only dependent upon the species and cell type examined, but also upon the nature of the surrounding ECM. Our studies also support the general idea that certain genes may contain specific 5′ flanking sequences that are ECM-responsive (reviewed in Jones et al., 1993; Chiquet-Ehrismann et al., 1994; Myers et al., 1998).

In conclusion, we have suggested a mechanism by which remodeling of type I collagen modulates TN-C gene transcription via a β3 integrin and MAPK-mediated signaling pathway, which represents a paradigm for vascular development and disease whereby SMC respond to perturbations in ECM composition by altering their phenotype and patterns of gene expression.

The authors would like to thank Dr Harold Erickson for providing anti-TN-C antisera, and Alexander Cheah and Claire Coulber for help in preparing reagents and the manuscript. This study was supported by program grant T2229 from the Heart and Stroke Foundation of Canada.

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